Broncho-alveolar lavage in acute rejection and lymphocytic bronchiolitis after lung transplantation: is neutrophilia a paradox or opportunity for tailored treatment?

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Abstract

Acute cellular rejection (grade A) and lymphocytic bronchiolitis (grade B) may impair allograft function after lung transplantation (LTx).

We investigated broncho-alveolar lavage (BAL) cellular and protein profiles for different grade A or B severity scores, hypothesizing that concurrent BAL neutrophilia might favor azithromycin-use in either condition.

All consecutive BAL with subsequent transbronchial biopsies (TBB), performed in 339 LTx recipients from 2001 to 2008, were retrospectively analyzed. TBB were classified according to histological grade and compared for BAL cell differentials and IL-6/IL-8/TGF-β protein levels.

We included 768 TBB for analysis and excluded 64 TBB with histological signs of infection. BAL total cell count significantly increased with grade A or B severity. A higher A grade was associated with a significant increase in lymphocytosis and to a lesser extent neutrophilia, whereas for higher B grades increased neutrophilia was present. IL-8 levels similarly were associated with grade A or B severity. A similar trend was seen for IL-6 with grade A and TGF-ß with grade B severity.

A distinct BAL profile is present with increasing grade A or B severity. Especially B grades are characterized by increased BAL neutrophilia; azithromycin therapy might thus be beneficial, which warrants further prospective investigation.

Abstract word count: 197 Keywords: acute rejection, biopsy, broncho-alveolar lavage, lung transplantation, lymphocytic bronchiolitis.

Introduction

Acute cellular rejection (AR, grade A) and lymphocytic bronchiolitis (LB, grade B) are major histological risk factors for bronchiolitis obliterans syndrome (BOS) and mortality after lung transplantation (LTx) (1-4). Fiber-optic bronchoscopy with transbronchial lung biopsies (TBB) remains the gold standard for diagnosis and both conditions are histologically graded according to intensity and distribution of mononuclear cellular infiltrates, either perivascular and interstitial (A0-none, A1-minimal, A2-mild, A3-moderate, A4-severe), or submucosal (B0-none, B1R (previous B1 and B2)-low grade, B2R (previous B3 and B4)-high grade, BX-upgradeable) (2). In higher A and B-grades (A3/4, B2R) some neutrophils may be present within the biopsy specimen, yet the presence of a disproportionate number of neutrophils in relation to mononuclear cells is highly suggestive of infection rather than rejection (2). Concurrent LB may be seen in association with AR grade A2 or greater, but is less common with minimal (grade A1) AR (2). Nevertheless, it has only recently been recognized that LB may have similar implications as AR for allograft function and the appearance of obliterative bronchiolitis (OB) after LTx (3;4). Treatment options for both AR and LB predominantly consist of high-dose systemic steroids, often in association with modifying the immunosuppressive regimen, however addition of inhaled steroids may also be beneficial in LB (5). Although broncho-alveolar lavage (BAL) fluid in AR has previously been shown to be characterized by marked (predominantly $CD8^+$) lymphocytosis (6-8), only very few studies actually correlated BAL findings with AR and/or LB-severity (8;9). Furthermore, during the past years novel anti-inflammatory and anti-oxidative drugs, such as azithromycin, have been introduced in the field of LTx, yet its potential benefit in AR or LB has thus far never been investigated.

The aim of the present study was therefore to investigate BAL cellular and protein profiles of particular pro-inflammatory cyto-/chemokines and remodeling factors (IL-6, IL-8, TGF-ß) in different AR or LB severity grades, as we hypothesized that there may be a discrepancy between AR and LB or different grades (particularly considering BAL neutrophilia), which could possibly favor response to azithromycin therapy in these conditions.

Materials and methods

Study design and population

The study comprises a retrospective analysis of all surveillance or clinically indicated consecutive bronchoscopic procedures with BAL and TBB, performed between Oct. 2001 and Oct. 2008, in a cohort of 339 LTx recipients (transplanted from Aug. 1991 to Sept. 2008). The evaluation was restricted to this 7-year period because since Oct. 2001 all LTx recipients are enrolled in a prospective bronchoscopic routine follow-up program in our center and because throughout the subsequent years the revised 1996 International Society for Heart and Lung Transplantation (ISHLT) classification for TBB was uniformly applied (10). The study was approved by the local University Hospital Ethical Review Board and all patients gave written informed consent.

Bronchoscopy with BAL and/or TBB

Bronchoscopic procedures, for microbiological and virological assessment, quantification of cell differentials and protein levels and/or histology, were performed as previously described (11). Briefly, BAL was routinely carried out around fixed time points after LTx (21, 90, 180, 360, 540, 720, 900, 1080, 1260, 1440, etc. days), or if AR, infection or BOS was clinically suspected. Likewise, surveillance TBB was routinely performed at days 21 and 90 post-LTx, thereafter in case of clinical suspicion of AR, infection or BOS.

BAL was performed in a subsegmental bronchus of the right middle lobe (bilateral or right single LTx) or lingula (left single LTx) with two 50 mL aliquots of sterile saline at room temperature. After each instillation, BAL fluid was recovered by manual suction and fractions were pooled for analysis. Subsequently, TBB were performed as outlined in the revised ISHLT guidelines (2) in right middle and/or lower lobe or left lower lobe if indicated.

Five mL of the BAL fluid was sent for microbiological and virological assessment, whereas the remaining fluid was immediately transported to the lab for further analysis, blinded to the patient's clinical status. A cytospin was made with 10^5 cells/mL in a Shandon cytocentrifuge

(Techgen, Zellik, Belgium) and stained with May-Grünwald-Giemsa. Differential cell counts were determined by counting at least 300 cells. If lymphocytosis was above 10%, lymphocyte sub-types (CD19+ B and CD3+T-cells with CD4+ and CD8+ count) were additionally analyzed using flow-cytometric analysis (monoclonal antibodies and FACSCanto, Becton Dickinson, Erembodegem, Belgium). BAL protein levels of IL-6, IL-8 and TGF-β were analyzed in duplicate in 100 µl undiluted BAL supernatant using commercially available sandwich enzyme-linked immunosorbent assays (ELISA) (Invitrogen, Merelbeke, Belgium). If the concentration was below the detection limit (respectively 0.2, 1 and 5 pg/mL), a value of half this limit was accorded for analysis.

TBB specimens were fixed in formalin, embedded into a paraffin block and subsequently 5 µm histological sections were obtained by serial cutting at two levels and immunohistochemical staining (Hematoxylin-Eosin as well as Gram staining and if indicated staining for *cytomegalovirus* (CMV), *Herpes*, *Pneumocystis*, as well as Verhoeff's Elastica, Ziehl-Neelson and/or Grocott staining) according to standard criteria. All TBB specimens were examined by a single pathologist (EKV) skilled in LTx, who was blinded to the patients' clinical status; and graded according to the 1996 ISHLT guidelines (grade A0-4 with concomitant B0-4) (10). 'Bx' biopsies were evaluable for A grading, but did not show bronchiolar epithelium to allow a B grade to be determined. Similarly, biopsies with bronchiolar tissue, but no or insufficient alveolated lung parenchyma, were classified as 'Ax' for the current study. 'Inadequate' biopsies (AxBx) did neither show adequate bronchiolar epithelium nor sufficient alveolar tissue for A or B grading, or demonstrated other interstitial lesions of the pulmonary graft (e.g. bronchiolitis obliterans organising pneumonia (BOOP), diffuse alveolar damage (DAD), interstitial pneumonitis). For the current study, TBB were classified according to A and/or B grade and subsequently compared for demographical and BAL characteristics.

Assessment of pulmonary function

Spirometry was performed in agreement with American Thoracic Society (ATS)-criteria prior to bronchoscopy (Masterscreen, Jaeger, Hoechberg, Germany) (12).

Immunosuppressive and prophylactic regimen

Conventional post-operative immunosuppressive and prophylactic treatment has been previously described (13, *online supplementary*). Concisely, all LTx recipients received triple-drug immunosuppressive therapy with methylprednisolone, azathioprine (AZA) or mycophenolate mofetil (MMF) and cyclosporine A (CsA) or tacrolimus (FK). AR grade >A1 was treated with intravenous pulse doses of methylprednisolone, tapered to the oral maintenance dose over the next 2-3 weeks, while A1 rejection was treated with augmenting oral steroids, similarly followed by tapering. Additionally, in case of recurrent AR, conversion from CsA to FK or from AZA to MMF was performed. There was no strict protocol for treatment of isolated grade B rejection, although most patients were treated similar to the protocol of grade A rejection by increasing steroids and/or changing immunosuppressives. Additionally, BOS ≥0-p was additionally treated with azithromycin and in case of progression with rapamycin, total lymph node irradiation or retransplantation. Initial infectious prophylaxis consisted of ganciclovir or acyclovir for CMV (depending on donor and recipient CMV status), inhaled amphotericin B for *Aspergillus* and long-term prophylaxis with sulfamethoxazole-trimethoprim for *Pneumocystis*.

Statistical analysis

For statistical analysis, every TBB specimen was regarded independently and investigated for concurrent clinical or BAL findings. Using Graphpad Prism 4.0 (San Diego, CA, USA), Wilcoxon signed rank test (*online supplement*), Mann-Whitney-U test or non-parametric Kruskall-Wallis with Dunn's post-testing were performed where appropriate. Contingency tables were analyzed using Chi square test. Correlation between severity of histological grade on TBB (0 to 3) and various clinical and BAL parameters was assessed by Spearman rank test. Results of

the data are presented either as total values, mean (±standard deviation), median (with inter-quartile range) or as percentages. A p-value of <0.05 was considered significant.

Results

The included patients' characteristics are summarized in table 1. A total of 1984 bronchoscopic procedures were performed in 339 LTx recipients, with a median of 6 (3-8) per patient and at a median of 182 (37-546) days after LTx. Subsequent TBB were performed in 832 cases (59.4% as surveillance and 40.6% on clinical indication), with a median of 2 (1-3) TBB per patient performed at a median of 92 (32-367) days after LTx. After exclusion of 64 (7.7%) TBB with histological signs of concurrent bacterial/fungal or CMV-infection (14;15), a total of 768 TBB with preceding BAL were included for the current analysis (Figure 1). Distribution of excluded and included TBB specimens according to A and B grading is presented in table 2. The yield of bronchoscopy to detect AR (grade A>0) or LB (grade B>0) in the included TBB specimens was respectively 28.6% (n=220/768) and 14.5% (n=111/768), which is within the previously reported range (16). As only one TBB demonstrated grade A4B4 (severe) rejection, this sample was not included for further analyses.

When histological grade A or B severity scores (0-3, *excluding* x) were correlated with concurrent clinical and BAL findings, not only an association between grade A and B severity scores could be demonstrated (r=0.20, p<0.0001), but also between the grade A, respectively B, severity score and other relevant parameters (table 3). Grade A severity, for instance, was inversely related with FEV_1 (% predicted), whereas for grade B severity no association was seen. On the other hand, grade B severity correlated with time of TBB sampling after LTx, whereas for grade A no association was present (table 3). Neither increasing grade A nor grade B severity was associated with the volume of BAL fluid return; however, both did correlate with BAL total cell count. Higher grade A, but not B, severity scores were associated with elevated BAL lymphocyte percentages (table 3). Particularly BAL $CD8^+$ T-cells tended to be associated with higher grade A

severity (r=0.15, p=0.08), whereas no correlation was seen with either BAL B-cells or $CD4^+$ T-cells (p=0.30 and p=0.12 respectively). Both higher grade A and B severity correlated with increased BAL neutrophil percentages (table 3). As expected by the increase in neutrophils and lymphocytes, macrophage percentages were inversely associated both with higher grade A or B severity. Eosinophilia, on the other hand, was associated with grade A, but not B, severity (table 3). IL-6 levels tended to be associated with grade A severity, but not with grade B severity, whereas IL-8 levels correlated with both. TGF-ß protein levels, on the other hand, were inversely related with grade A severity and tended to be directly associated with higher B-grade severity (table 3). No correlation whatsoever was seen between severity of histological A or B grade and either the presence of gram-negative or –positive bacteria in BAL, or concurrent azithromycin-use at the time of sampling (table 3). Reanalysis after excluding TBB with concurrent azithromycin-use (n=120/768, 15.6%) revealed similar significant associations (data not shown).

Because Ax and Bx TBB samples were not included in correlation-analyses with grade A or B severity scores (i.e. 0-3); variations between different grade A severity scores (regardless of concurrent grade B severity: A0-3B0/1/2/3/x), or between grade B severity scores (regardless of concurrent grade A severity: B0-3A0/1/2/3/x) respectively, were additionally analyzed and summarized in table 4 and figure 2. Similar associations between increasing grade A (regardless of grade B) or B (regardless of grade A) severity scores and the various clinical and BAL cellular characteristics mentioned before were seen when taking Bx, respectively Ax, samples into account (table 4, figure 2).

Next, excluding a possible confounding effect of concurrent grade B or A rejection, subanalysis of BAL total cell count and cell differentials (neutrophilia and lymphocytosis) was performed for each grade A (A0/1/2/3/x) in absence of concurrent grade B rejection (B0) and for each grade B (B0/1/2/3/x) in absence of concurrent grade A rejection (A0) respectively (Table 4, first column, figure 3A). Within both groups (i.e. A0B0/1/2/3/x, B0/A0/1/2/3/x) a similar pattern regarding BAL total cells, neutrophilia and lymphocytosis could be demonstrated with increasing

grade B, respectively A severity (figure 3A). The observed cellular differences again were independent form the presence of gram-negative or -positive bacteria in BAL or concurrent azithromycin-use at the time of sampling (figure 3A). Furthermore, BAL neutrophilia tended to be more severe in the higher B when compared to A grades (A0B1 vs. A1B0 p=0.05, A0B2 vs. A2B0 p=0.07 and A0B3 vs. A3B0 p=0.08 respectively), whilst for lymphocytosis a similar pattern (however also not statistically significantly different) was seen for the highest A compared to B grades (figure 3B).

While assessing the aforementioned data, we additionally identified a historical cohort of 8 LTx recipients in whom LB was diagnosed *without* concurrent grade A-rejection, signs of infection or OB/BOS and who (all except one) initially had been treated by augmenting steroids, yet because of unsatisfactory FEV_1-recovery soon thereafter were empirically started on azithromycin (figure 3, *online supplement*). Despite the fact of being a historical cohort and lacking placebo-treated controls, the additional significant improvement in FEV_1 and decrease in BAL neutrophilia observed in these patients thereafter were at least impressive especially when compared with a matched, azithromycin-naïve cohort (figure in *online supplement*).

Discussion

This is the first study to date demonstrating a direct relationship between BAL cellular and protein characteristics with the severity of either grade A and B rejection score of concurrent TBB specimens. Increasing grade A severity (regardless of concurrent grade B severity) was associated both with an increase of BAL lymphocytosis and to a lesser extent neutrophilia, whereas for the higher B grades (regardless of concurrent grade A severity) particularly marked neutrophilia was present. IL-8 protein levels showed a similar association with both grade A or B severity. A similar trend was seen for IL-6 with grade A and for TGF-ß with grade B severity. Furthermore, even in absence of concurrent grade B rejection, grade A severity seems to be associated with BAL lymphocytosis, as previously demonstrated (6), whereas grade B severity in absence of concurrent grade A rejection is more related to BAL neutrophilia, as was already suggested by Zheng *et al*. (7). The current data may also confirm the previous report by Tikkanen *et al.* demonstrating increased proportions of BAL lymphocytes in 'early' versus neutrophils in 'later' (i.e. >180 days post-LTx) occurring rejection, although their use of a semi-quantitative scoring system of TBB specimens makes it impossible to know whether the latter condition specifically corresponds with B grades or the more severe rejections of the present ISHLT scoring system in general (15). Similarly, a recent gene expression study of BAL cells in acute rejection (defined as a combined A+B score ≥2) by Patil *et al.* demonstrated an increase of both BAL lymphocyte and particularly neutrophil percentages with increasing probability of rejection (generated by means of 'prediction analysis of microarrays' based on BAL gene expression profile) (17). Yet again, merging histological grade A and B scores results in a less specific scoring; which in fact makes it impossible to assess BAL cellularity according to separate grade A or B severity scores, as was performed in our study. Nevertheless, our data, together with that of the above-mentioned groups, are corroborated by a recent study in renal transplant recipients demonstrating that biopsy specimens in acute rejection may reveal extensive differences in gene expression, which are compatible with different subtypes of acute rejection characterized by

differences in immunologic and cellular features, but also in clinical course (e.g. glucocorticoid resistance, graft loss) (18). The most striking observation in our study, however, may be the apparent paradox in histological and concurrent BAL findings in LB showing mononuclear lymphocytic cellular infiltrates in the airway wall on the one hand and a predominant luminal (BAL) neutrophilia on the other hand. Moreover, these findings were not only present in higher B-grades (B3/4 or B2R), in which some neutrophils may also be present within the biopsy specimen as stated in the revised ISHLT scoring system (2), but also in the lower grades (B1/2 or B1R), possibly indicating a specific characteristic of LB. As BAL samples and TBB represent cells from different compartments of the broncho-alveolar unit, some discrepancies between both may be present due to different biological events in the airway lumen compared to the wall, but it seems highly unlikely this can explain the observed differences in cellularity and protein levels between various grade A or B severities.

Despite the fact that LB was already recognized in pulmonary allografts almost two decades ago (9), its importance was left for a long time undetermined in the ISHLT allograft rejection working formulation (10). Moreover, its definition has considerably changed with successive revisions of the ISHLT classification, further obscuring assessment of its significance after LTx (2). In recent years, however, it has become clear that LB is an independent risk factor for subsequent development of BOS (19), which interestingly even seems to be directly related to the severity of LB (3;4). Since BAL neutrophilia is considered as a hallmark of the azithromycin-responsive phenotype of BOS (20), together with both the fact that LB seems to appear later after LTx (21) and that increasing severity of LB is associated with more pronounced BAL neutrophilia (as shown herein), one could question the current dogma of LB being a marker of ‘acute’ allograft rejection. It may therefore perhaps be more appropriate to be considered as an early stage in the evolution of chronic allograft dysfunction, in particular the neutrophilic reversible allograft dysfunction BOS-phenotype.

As clinical diagnosis is often difficult, due to confounding (or even little or no) symptoms, various or non-specific radiographic abnormalities and inconsistent spirometric results, many centers have implemented surveillance bronchoscopy with TBB (despite its rather low diagnostic yield) and institute pre-emptive therapy for AR and LB, hoping to prevent evolution to chronic rejection. Yet, both histological and associated pulmonary function changes in either condition may be refractory to treatment, in particular corticosteroids, suggesting that perhaps also alternative therapies should be implemented (3;9;22). Therefore, keeping the current results in mind, 'tailored treatment' may be more appropriate by adding azithromycin in those cases expressing an increased BAL neutrophilia versus steroids alone for those with a predominant lymphocytosis. Although the anti-inflammatory and anti-oxidative effects of macrolides have been well established in patients with BOS since their introduction in the field of LTx, the potential benefit in AR or LB has thus far never been investigated. Yet, the subsequent evolution of FEV_1 and BAL neutrophilia in the historical cohort additionally described in the *online supplement* supports the former hypothesis that, particularly in LB, adding azithromycin may be beneficial. These preliminary observations of course remain to be confirmed in a formal, prospective placebo-controlled study.

Evidently, possible confounding factors need to be addressed concerning the current study. The main limitation of the present study remains it's retrospective, cross-sectional and monocentric design, but it is strengthened by the large number of biopsies taken, as well as the constancy of examination and reporting by a single experienced transplant pathologist over a 7-year period. However TBB specimens with obvious histological signs of infection were excluded for analysis, subclinical infection or airway colonization might nevertheless interfere with the interpretation of histological and concurrent BAL findings. Therefore, the presence of either Gram-negative or –positive bacteria in concomitant BAL fluid was also taken into account in the present study; which, however, could not explain the current findings. On the other hand, we acknowledge that reliance on BAL fluid cultures may not be that unfailing in immunosuppressed

LTx recipients because of the possibility of atypical or difficult-to-culture respiratory pathogens. Furthermore, it remains unclear whether the observed differences in BAL profile with increasing histological grade A or B severity may have been obscured by different immunosuppressives at the time of sampling (23). Next, as gastroesophageal reflux was not systematically assessed during the follow-up period of the studied cohort, its importance for BAL and histology could unfortunately not be investigated, as it has recently become increasingly obvious that reflux may be associated with airway inflammation (24). Finally, the presence of other interstitial lesions of the pulmonary graft, the role of the previously described T_H17-lineage (11;25) or the assessment of patient survival and freedom from BOS according to preceding grade A or B-rejection scores were not within the scope of the present paper.

In conclusion, this study is the first to demonstrate a direct association between the histological grade of either AR or LB with concomitant BAL cellular and protein characteristics. Higher grade A severity is mainly associated with increased BAL lymphocytosis, whereas both with increasing grade A, but particularly grade B, severity elevated BAL neutrophilia is present. Consequently, BAL findings may guide treatment, whereas specifically in LB there may be a benefit of additional azithromycin treatment (as is also suggested by the additional historical patient cohort in the *online supplement*). These preliminary observations, however, remain to be confirmed in a formal, prospective placebo-controlled study.

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Tables

Table 1: Included patients' characteristics

	LTx-procedures (n=351)
Age at LTx (years)	52 (38-58)
Gender (male/female)	189/162
Type of LTx [*]	
BLTx	240
SLTx	89
HLTx	22
Pre-LTx diagnosis	
Emphysema (COPD)[†]	145
Pulmonary Fibrosis	64
Cystic Fibrosis	51
α1-antitrypsin deficiency	22
Pulmonary Arterial Hypertension	22
Eisenmenger syndrome	12
Obliterative bronchiolitis	15
Bronchiectasis	10
Lymphangioleiomytosis	3
Histiocytosis X	2
Kartagener syndrome	2
Miscellaneous	3
Time of follow-up (years)	2.7 (1.3-5.4)

Table 1 legend

Characteristics for all included lung transplant (LTx) recipients (n=339), in whom 351 transplantations were performed (including 12 re-LTx). Data are presented as total value or median (inter-quartile range).

Abbreviations: [*]BLTx: bilateral lung transplantation, SLTx: single lung transplantation, HLTx: heart-lung transplantation, [†]COPD: chronic obstructive pulmonary disease.

Table 2: Distribution of TBB specimens according to A and B grading

Excluded, n (%)	A0	A1	A2	A3	A4	Ax	Total
B0	36 (56.3)	3 (4.7)	4 (6.3)	1 (1.6)	0	2 (3.1)	46 (71.9)
B1	2 (3.1)	0	0	0	0	0	2 (3.1)
B2	2 (3.1)	0	0	0	0	1 (1.6)	3 (4.7)
B3	1 (1.6)	0	1 (1.6)	0	0	0	2 (3.1)
B4	0	0	0	0	0	0	0
Bx	4 (6.3)	0	2 (3.1)	0	0	5 (7.8)	11 (17.2)
Total	45 (70.3)	3 (4.7)	7 (10.9)	1 (1.6)	0	8 (12.5)	64 (100.0)

Included, n (%)	A0	A1	A2	A3	A4	Ax	Total
B0	383 (49.9)	80 (10.4)	44 (5.7)	13 (1.7)	0	32 (4.2)	552 (71.9)
B1	31 (4.0)	16 (2.1)	12 (1.6)	2 (0.3)	0	3 (0.4)	64 (8.3)
B2	16 (2.1)	3 (0.4)	9 (1.2)	1 (0.1)	0	2 (0.3)	31 (4.0)
B3	6 (0.8)	2 (0.3)	2 (0.3)	3 (0.4)	0	2 (0.3)	15 (2.0)
B4	0	0	0	0	1 (0.1)	0	1 (0.1)
Bx	61 (7.9)	17 (2.2)	8 (1.0)	7 (0.9)	0	12 (1.6)	105 (13.7)
Total	497 (64.7)	118 (15.4)	75 (9.8)	26 (3.4)	1 (0.1)	51 (6.6)	768 (100.0)

Table 2 legend

Distribution of excluded and included transbronchial biopsy (TBB) specimens according to A and B-grading (10). Data are presented as total value (percentage).

Table 3: Correlation of grade A or B severity scores (0-3, *excluding* x) with concurrent clinical and BAL findings

		A0-3 (n=716)		B0-3 (n=662)	
		r	p	r	p
POD	(days)*	-0.06	0.10	0.09	**0.02**
FEV_1	(%pred)†	-0.17	**<0.0001**	-0.04	0.39
BAL	Return (mL/100mL)‡	-0.05	0.22	-0.05	0.26
	Total cells (x10³/mL)	0.16	**<0.0001**	0.21	**<0.0001**
	Neutrophils (%)	0.18	**<0.0001**	0.27	**<0.0001**
	Lymphocytes (%)	0.17	**<0.0001**	0.03	0.45
	Macrophages (%)	-0.24	**<0.0001**	-0.27	**<0.0001**
	Eosinophils (%)	0.15	**0.0001**	0.01	0.96
	IL-6 (pg/mL)§	0.07	0.08	-0.05	0.22
	IL-8 (pg/mL)§	0.13	**0.0008**	0.08	**0.04**
	TGF-β (pg/mL)‖	-0.10	**0.007**	0.07	0.08
	GN-culture positive, n (%)**	-0.03	0.44	0.03	0.48
	GP-culture positive, n (%)††	0.02	0.68	0.01	0.75
Azithromycin-use at TBB, n (%)‡‡		0.04	0.31	0.04	0.35

Table 3 legend

Transbronchial biopsies (TBB) classified according to their histological grade (0=absent, 1=minimal, 2=mild, 3=moderate, x=upgradeable) and subsequently compared for time of sampling, pre-bronchoscopy FEV_1 and concurrent BAL cellular and cytokine-profiles.

Ax (n=51/768 or 6.6%) and Bx (n=105/768 or 13.7%) TBB samples could not be included in correlation-analyses with grade A or B severity scores (0-3) and as only one TBB demonstrated grade A4B4 (severe) rejection, this sample was not included for analysis. Correlation coefficient r and p-value are given for non-parametric Spearman rank test.

Abbreviations: *POD=post-operative day after transplantation, †FEV_1=forced expiratory volume in one second (% predicted), ‡BAL= broncho-alveolar lavage, §IL= interleukin, ‖TGF-β= transforming growth factor-β, **GN= Gram-negative, ††GP= Gram-positive, ‡‡Azithromycin= concurrent treatment with azithromycin at the moment of transbronchial biopsy (TBB).

Table 4: Demographical and BAL characteristics according to TBB grade A or B severity score

		A0 B0/1/2/3/x n=497	A1 B0/1/2/3/x n=118	A2 B0/1/2/3/x n=75	A3 B0/1/2/3/x n=26	p	Ax B0/1/2/3/x n=51
POD	**(days)***	92.0 (37.0-376.5)	87.5 (26.5-403.5)	87.0 (24.0-278.0)	184.5 (36.0-444.5)	0.15	94.0 (92.0-645.0)
FEV_1	**(%pred)†**	65 (53-80)	59 (47-75) *	55 (43-70) ***	63 (44-79)	**0.001**	66 (50-86)
BAL	**Return (mL/100mL)‡**	40.0 (32.0-50.0)	43.0 (35.0-50.0)	38.0 (25.0-45.0)	36.0 (30.0-46.0)	0.06	42.0 (31.0-50.0)
	Total cells (x10³/mL)	140.0 (65.0-303.0)	195.5 (95.0-411.5) *	200.0 (98.0-606.5) **	288.5 (70.0-730.0) *	**0.0005**	133.0 (73.0-338.0)
	Neutrophils (%)	5.0 (1.6-25.5)	6.2 (1.6-40.0)	24.0 (5.5-61.9) ***	27.8 (7.1-81.5) ***	**<0.0001**	8.5 (1.4-49.5)
	Lymphocytes (%)	3.6 (1.5-7.6)	4.5 (2.0-10.5)	6.0 (3.0-10.8) **	6.2 (3.5-22.3) **	**<0.0001**	3.2 (1.6-7.6)
	Macrophages (%)	86.6 (62.4-94.2)	82.0 (51.8-92.4) *	60.0 (27.4-81.8) ***	32.3 (10.3-78.0) ***	**<0.0001**	81.4 (44.3-94.1)
	Eosinophils (%)	0.0 (0.0-0.2)	0.0 (0.0-0.2)	0.0 (0.0-0.5)	0.7 (0.0-2.2) ***	**<0.0001**	0.0 (0.0-0.5)
	IL-6 (pg/mL)§	0.4 (0.1-1.5)	0.4 (0.1-1.6)	0.5 (0.1-2.0)	0.8 (0.4-13.2) *	**0.016**	0.3 (0.1-1.6)
	IL-8 (pg/mL)§	42.5 (14.3-111.7)	46.7 (18.0-119.0)	94.2 (35.7-168.2) **	113.9 (30.3-189.8) *	**0.0009**	43.7 (13.7-133.9)
	TGF-β (pg/mL)‖	9.6 (2.5-117.8)	2.5 (2.5-63.7)	2.5 (2.5-68.2)	2.5 (2.5-68.5)	0.09	3.3 (2.5-115.0)
	GN-culture positive, n (%)**	87 (17.5)	18 (15.3)	10 (13.3)	5 (19.2)	0.77	11 (21.6)
	GP-culture positive, n (%)††	40 (8.0)	8 (6.8)	9 (12.0)	2 (7.7)	0.64	3 (5.9)
Azithromycin-use at TBB, n (%)‡‡		71 (14.3)	22 (18.6)	8 (10.7)	8 (30.8) *	0.06	11 (21.6)

		B0 A0/1/2/3/x n=552	B1 A0/1/2/3/x n=64	B2 A0/1/2/3/x n=31	B3 A0/1/2/3/x n=15	p	Bx A0/1/2/3/x n=105
POD	**(days)***	91.0 (30.5-339.0)	111.5 (27.0-657.5)	181.0 (72.0-538.0)	466.0 (179.0-997.0) *	**0.009**	91.0 (31.0-267.5)
FEV_1	**(%pred)†**	63 (50-79)	60 (44-80)	68 (47-75)	61 (46-70)	0.83	65 (53-77)
BAL	**Return (mL/100mL)‡**	41.0 (32.0-50.0)	40.0 (31.0-45.5)	40.5 (31.0-45.0)	36.5 (31.5-42.0)	0.35	40.0 (30.0-49.0)
	Total cells (x10³/mL)	142.5 (69.0-298.0)	218.5 (90.0-440.0)	506.0 (185.0-865.0) ***	598.0 (232.0-1489.0) ***	**<0.0001**	141.0 (70.0-350.0)
	Neutrophils (%)	5.0 (1.6-25.1)	20.0 (3.3-60.5) **	45.1 (12.2-76.2) ***	73.2 (42.6-83.6) ***	**<0.0001**	6.8 (1.8-32.0)
	Lymphocytes (%)	4.0 (1.6-8.0)	4.0 (2.0-10.4)	4.5 (1.8-9.6)	4.5 (2.1-12.0)	0.87	3.8 (1.6-8.4)
	Macrophages (%)	86.2 (60.5-94.2)	72.0 (34.5-90.5) **	40.7 (14.4-79.4) ***	21.8 (10.4-53.5) ***	**<0.0001**	84.0 (51.0-92.7)
	Eosinophils (%)	0.0 (0.0-0.2)	0.0 (0.0-0.2)	0.0 (0.0-1.1)	0.0 (0.0-1.0)	0.37	0.0 (0.0-0.3)
	IL-6 (pg/mL)§	0.4 (0.1-1.6)	0.4 (0.1-1.6)	0.1 (0.1-1.4)	0.6 (0.1-2.2)	0.40	0.4 (0.1-1.5)
	IL-8 (pg/mL)§	42.8 (14.3-116.4)	52.9 (21.9-156.9)	80.8 (17.3-163.6)	74.6 (10.1-194.3)	0.22	51.2 (21.0-159.0) *
	TGF-β (pg/mL)‖	2.5 (2.5-99.7)	25.2 (2.5-134.1)	5.8 (2.5-63.9)	57.3 (11.1-235.8)	0.07	2.5 (2.5-105.9)
	GN-culture positive, n (%)**	94 (17.0)	10 (15.6)	6 (19.4)	5 (33.3)	0.24	16 (15.2)
	GP-culture positive, n (%)††	43 (7.8)	8 (12.5)	2 (6.5)	0 (0.0)	0.40	9 (8.6)
Azithromycin-use at TBB, n (%)‡‡		90 (16.3)	13 (20.3)	3 (9.7)	6 (40.0)	0.06	8 (7.6)

Table 4 legend

Transbronchial biopsies (TBB) classified according to their histological grade (0=absent, 1=minimal, 2=mild, 3=moderate, x=upgradeable) and subsequently compared for time of sampling, pre-bronchoscopy FEV_1 and concurrent BAL cellular and cytokine-profiles. As only one TBB demonstrated grade A4B4 (severe) rejection, this sample was not included for comparison.

Variations between grades A0-3B0/1/2/3/x and B0-3A0/1/2/3/x respectively were calculated by non-parametric Kruskall-Wallis test with Dunn's multiple comparison test for post-testing (with significances compared to A0B0/1/2/3/x or B0A0/1/2/3/x respectively expressed as * $p<0.05$, **: $p<0.01$, ***: $p<0.001$). Chi-square test was used to compare proportions between groups. Additionally, the groups AxB0/1/2/3/x and BxA0/1/2/3/x were also compared with the group with the lowest histological A or B grade (A0B0/1/2/3x or B0A0/1/2/3/x respectively), significances were analyzed by Mann-Whitney test and expressed as *: $p<0.05$. Data are presented as median (inter-quartile range).

Note:

- BAL cell count and differentials (neutrophilia en lymphocytosis) between different groups are also presented in figure 2.

- Data of the lowest histological A or B grade (A0B0/1/2/3x or B0A0/1/2/3/x respectively) within the first column were further analyzed for BAL cell count and differentials (neutrophilia and lymphocytosis) and are presented in figure 3A+B.

Abbreviations: [*]POD=post-operative day after transplantation, [†]FEV_1=forced expiratory volume in one second (% predicted), [‡]BAL= broncho-alveolar lavage, [§]IL= interleukin, [‖]TGF-β= transforming growth factor-β, [**]GN= Gram-negative, [††]GP= Gram-positive, [‡‡]Azithromycin= concurrent treatment with azithromycin at the moment of transbronchial biopsy (TBB).

Figure Legends

Figure 1: Study design

Abbreviations: LTx=lung transplantation, TBB=transbronchial biopsy, BAL=broncho-alveolar lavage, CMV=cytomegalovirus, A=acute rejection and B=lymphocytic bronchiolitis (according to histological grade 0=absent, 1=minimal, 2=mild, 3=moderate, 4=severe, x=upgradeable).

Figure 2: BAL cellular characteristics according to grade A or B severity score

Transbronchial biopsies (TBB) were classified according to their histological grade (0=absent, 1=minimal, 2=mild, 3=moderate, x=upgradeable) and subsequently compared for BAL cellular profiles. As only one TBB demonstrated grade A4B4 (severe) rejection, this sample was not included for comparison.

Variations between grades A0-3B0/1/2/3/x, respectively B0-3A0/1/2/3/x, were calculated by non-parametric Kruskall-Wallis test with Dunn's post-testing (with significances compared to A0B0/1/2/3/x or B0A0/1/2/3/x respectively expressed as * $p<0.05$, **: $p<0.01$ or ***: $p<0.001$). Bars represent mean+SEM.

Note: data of the lowest histological A or B grade (A0B0/1/2/3x or B0A0/1/2/3/x respectively) were further analyzed for BAL cell count and differentials (neutrophilia and lymphocytosis) and are presented in figure 3A+B.

Figure 3: Subanalysis of BAL cellular characteristics according to grade A or B severity score

Panel A (top): Transbronchial biopsies (TBB) were classified according to their histological grade (0=absent, 1=minimal, 2=mild, 3=moderate, x=upgradeable) and subsequently compared for BAL cellular profiles. As only one TBB demonstrated grade A4B4 (severe) rejection, this sample was not included for comparison.

Variations between grades A0B0/1/2/3 or B0A0/1/2/3 respectively were calculated by non-parametric Kruskall-Wallis test with Dunn's post-testing (with significances compared to A0B0 or B0A0 respectively expressed as * $p<0.05$, **: $p<0.01$ or ***: $p<0.001$). Bars represent mean+SEM. Abbreviations: BAL=broncho-alveolar lavage, GN=presence of Gram-negative bacteria in BAL (n-value or %), GP=presence of Gram-positive bacteria in BAL (n-value or %), AZI=concurrent treatment with azithromycin at the moment of transbronchial biopsy (TBB) (n-value or %).

Panel B (bottom): Comparison of BAL neutrophilia and lymphocytosis according to histological A or B severity grade, in absence of concurrent grade B or A rejection respectively. Differences between grades (A0B1 *vs.* B0A1, A0B2 *vs.* B0A2 and A0B3 *vs.* B0A3) were analyzed by Mann-Whitney test and $p<0.05$ was considered significant. Data are represented as mean+SEM.

Figures

Figure 1

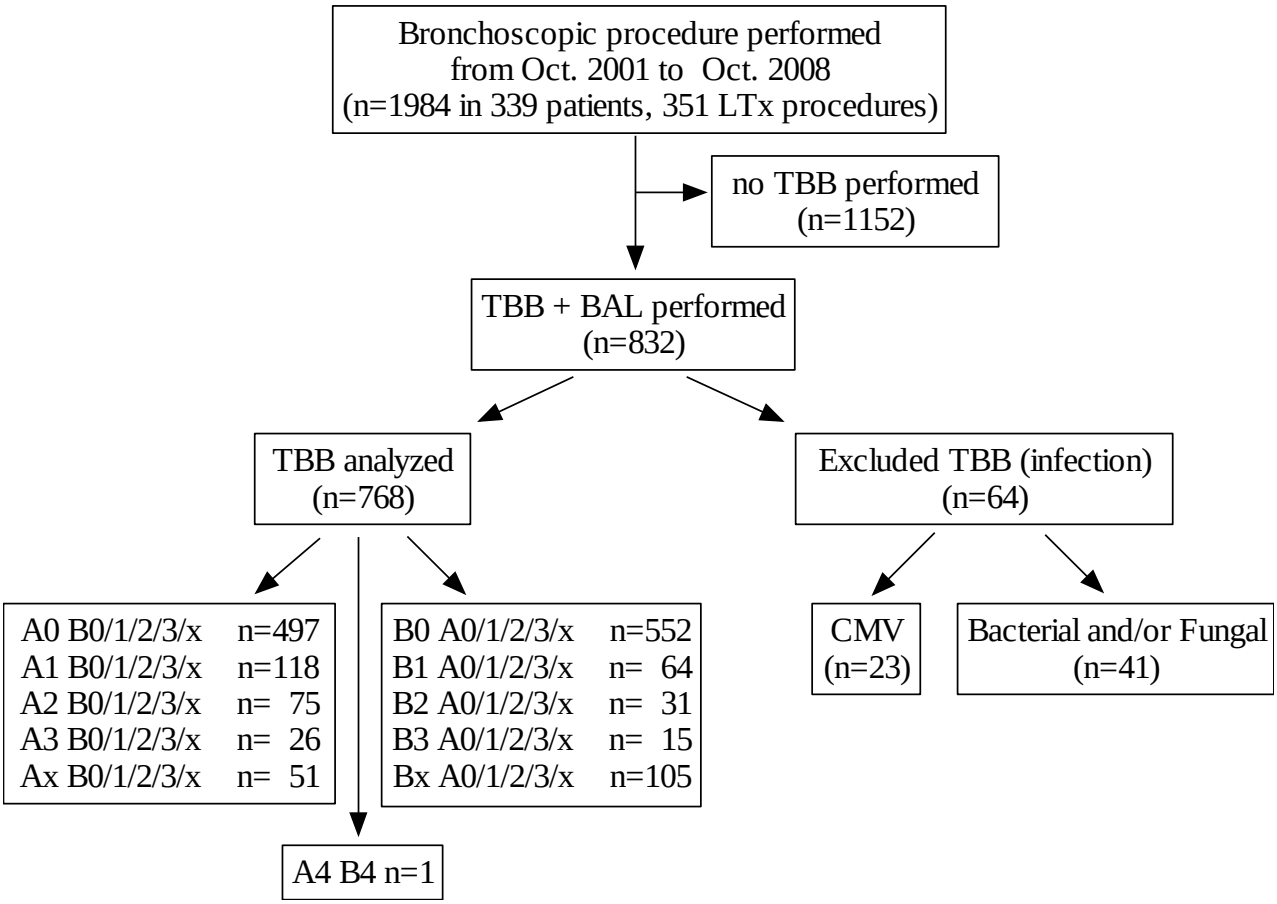
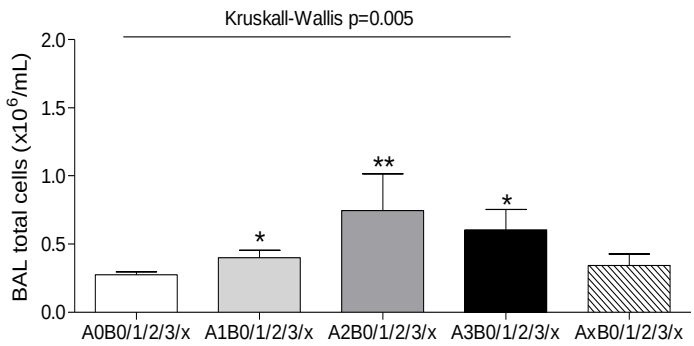
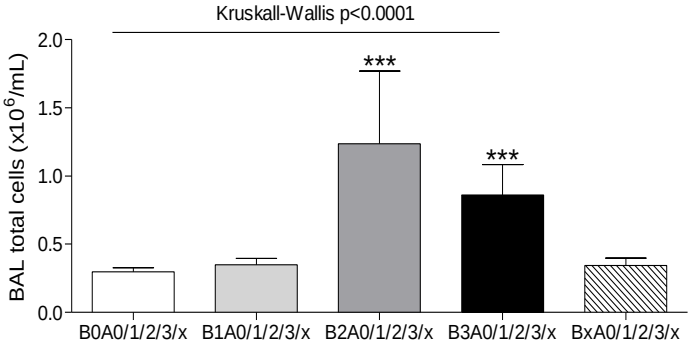
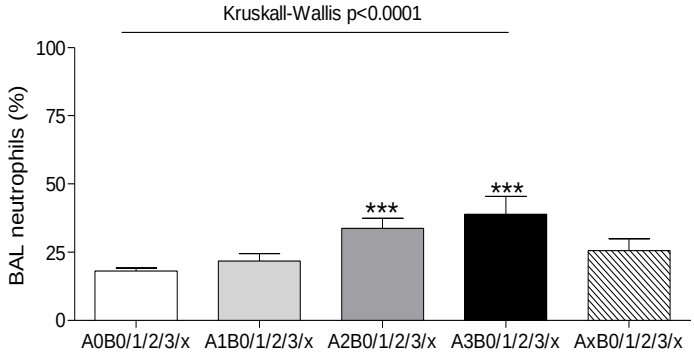
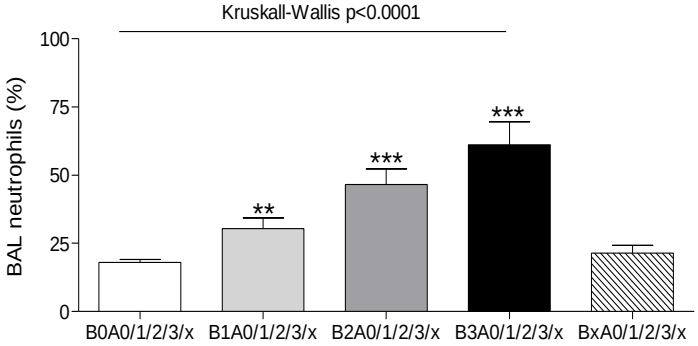


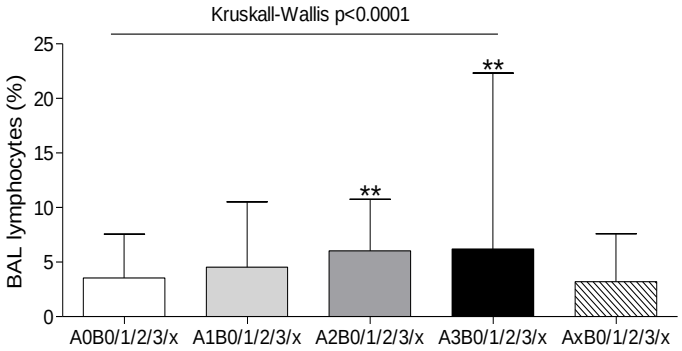
Figure 2

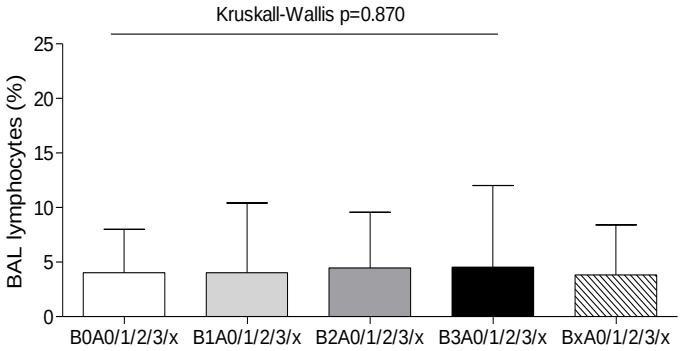






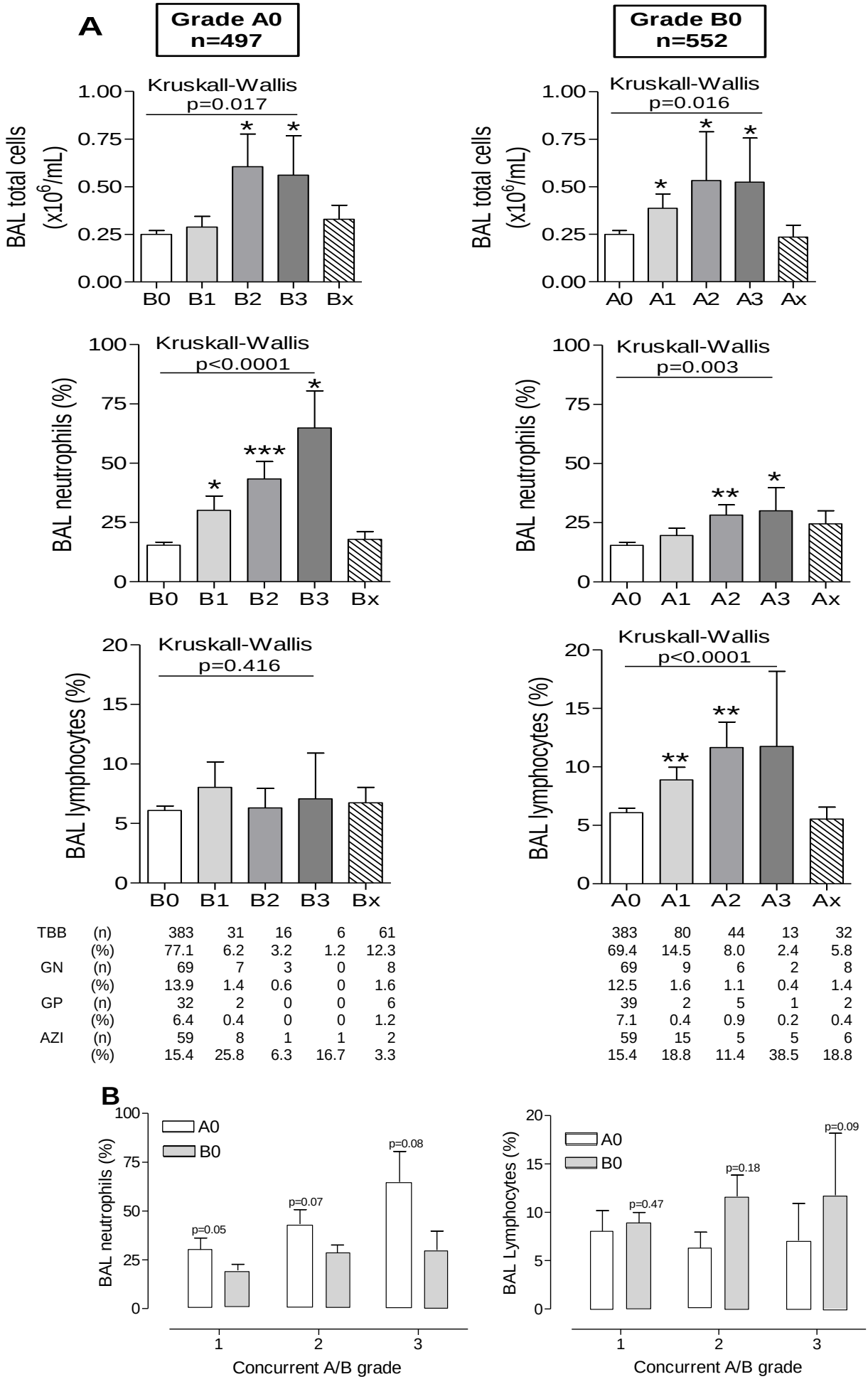






TBB (n)	497	118	75	26	51	552	64	31	15	105
(%)	64.7	15.4	9.8	3.4	6.6	71.9	8.3	4.0	2.0	13.7

Figure 3


A
Grade A0
n=497
Grade B0
n=552
Kruskall-Wallis
p=0.017
Kruskall-Wallis
p=0.016
BAL total cells
(x10^6/mL)
1.00
0.75
0.50
0.25
0.00
B0 B1 B2 B3 Bx
A0 A1 A2 A3 Ax
Kruskall-Wallis
p<0.0001
Kruskall-Wallis
p=0.003
BAL neutrophils (%)
100
75
50
25
0
Kruskall-Wallis
p=0.416
Kruskall-Wallis
p<0.0001
BAL lymphocytes (%)
20
15
10
5
0
TBB (n) 383 31 16 6 61
(%) 77.1 6.2 3.2 1.2 12.3
GN (n) 69 7 3 0 8
(%) 13.9 1.4 0.6 0 1.6
GP (n) 32 2 0 0 6
(%) 6.4 0.4 0 0 1.2
AZI (n) 59 8 1 1 2
(%) 15.4 25.8 6.3 16.7 3.3
383 80 44 13 32
69.4 14.5 8.0 2.4 5.8
69 9 6 2 8
12.5 1.6 1.1 0.4 1.4
39 2 5 1 2
7.1 0.4 0.9 0.2 0.4
59 15 5 5 6
15.4 18.8 11.4 38.5 18.8
B
A0
B0
p=0.05
p=0.07
p=0.08
p=0.47
p=0.18
p=0.09
1
2
3
Concurrent A/B grade

Supplementary material

FEV_1 and BAL neutrophilia in two matched historical patient cohorts

Lymphocytic bronchiolitis (A0B1 n=3, A0B2 n=2, A0B3 n=3; *without* signs of infection or OB/BOS) was diagnosed on transbronchial biopsies (TBB) at a median of 115 (68-631) days after LTx in 8 LTx recipients (Single (S) LTx =n1, Bilateral (B) LTx n=7) (panel A). Mean BAL neutrophilia at TBB was 68.0±20.4% (n=8), one BAL sample was colonized with *P. aeruginosa* and one with *S marcescens*.

One patient (A0B1) received no subsequent treatment, whilst the other were treated with augmenting methylprednisolone (IV 3x500mg/d n=6 or PO 3x48mg/d n=1, followed by tapering). Because of persistent dyspnea, coarse crackles on auscultation and insufficient improvement of FEV_1 (mean 5.5±6.1% or 190±224mL, p=0.11) at re-evaluation 11 (10-19) days after TBB, azithromycin (AZI) was associated (250 mg/2d) after which FEV_1 further significantly improved with 13.5±7.4% or 414±236mL (p=0.008) (panel B) and BAL neutrophilia decreased to 13.7 (±11.7)% (n=6, p=0.03) 3 months (mo.) later (panel C). In a matched, historical patient-cohort (similar age, type of LTx, timing and grade of TBB; p-value calculated by χ^2 or Mann-Whitney-U test; panel A), of which all but one were similarly treated by augmenting methylprednisolone at the time of TBB (IV n=6, PO n=1), but none with subsequent azithromycin, a lesser (however not statistically significant) improvement in FEV_1 and decrease in BAL neutrophilia was seen.

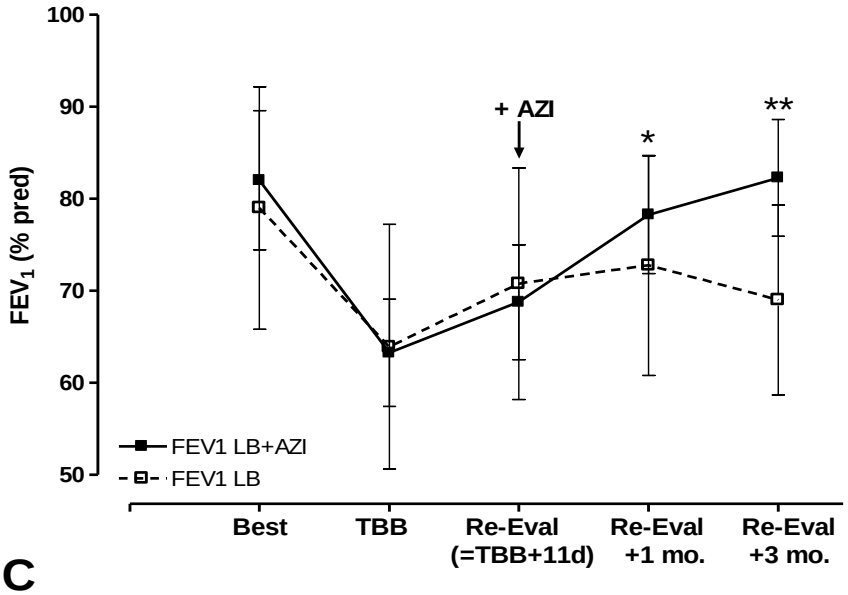
Data in graph are expressed as mean±SEM, * $p<0.05$, ** $p<0.01$ (Wilcoxon Signed Rank test vs. start of AZI for FEV_1 or vs. BAL at TBB for cell counts).

Online supplement Figure

A

	LB+AZI	LB	p-value
Male/Female	5/3	6/2	0.865
Type of LTx (S/B)	1/7	1/7	/
Age (years)	45.5 (+/-12.7)	51.7 (+/-12.0)	0.328
Diagnosis			0.973
Emphysema	4	4	
Fibrosis	1	2	
Cystic Fibrosis	1	0	
Bronchiectasis	1	1	
LAM	1	0	
OB	0	1	
Time of TBB (days)	115 (68-631)	188 (51-474)	0.789
TBB grade	A0B1 n=3	A0B1 n=3	
	A0B2 n=2	A0B2 n=2	
	A0B3 n=3	A0B3 n=3	

B



C